IMAGE BASED TRANSCRIPTOMICS EXPERIMENTAL PROTOCOL WWW.PELKMANSLAB.ORG

This protocol allows image-based transcriptomics as described by Battich, Stoeger et al. 2013 (Nature Methods, Image-based transcriptomics in thousands of single human cells at single-molecule resolution).

When setting up your equipment, run several plates on several different days and biological replicates and monitor multiple wells in the same plates. Only this way you will be able to exclude technical problems. If the absolute spot number is not reproducible you have a problem in your assay (e.g.: cell seeding, FISH protocol or data analysis).

EQUIPMENT

- **A proper incubator is the most important requirement for the assay as it prevents temperature gradients across the plate.** We strongly recommend a Liconic incubator. Load the plate by the automated stage and keep the plates cycling during the assay. You will have positional effects between multiple wells of a plate (up to 50% reduced spot number in inner wells!), if you use a normal tissue culture incubator or if you open the front door of the Liconic or if you use a heating block placed inside an tissue culture incubator or if you have the plates swimming in a water bath (where air cushions will form at the side of the plate).
- Pipetting robot. While it is tempting to skip the in-solution mixing steps, this mixing is essential for homogeneous and reproducible staining results.
- Washing Robot: We recommend a Biotek EL406. Use peristaltic pumps for all solutions except for PBS, which you can dispense by the syringe. (We do not use the dispenser of the manifold).
- Microscope: Ensure that illumination is stable during the scanning of a plate. For high-throughput we recommend a Yokogawa CV7000 microscope equipped with multiple large sCMOS cameras for simultaneous acquisition. A 40x objective (and even a 20x objective) is sufficient for the quantification of the majority of all genes (Battich et al. 2013)
- **Use branched DNA single-molecule FISH reagents** (e.g.: www.panomics.com).

ENSURE REPRODUCIBILITY

- Have a highly reproducible and tested cell seeding protocol. Ensure that there is no positional bias towards the border of a plate or for cells within a well. We follow the protocol of Lundholt et al. 2003 (JBS: A simple technique for reducing edge effect in cell-based assays) to prevent edge effects and tested different seeding volumes and dispensing heights to ensure even spread of cells within single wells. **Changes of cellular physiology will affect gene expression.**
- Make sure that all equipment is clean. Always clean the manifolds, the carrier of the aspirator and peristaltic pumps 2-3 days prior to the assay. Check calibration after cleaning.
- Ensure that the equipment is properly calibrated (2-3 days prior assay). If calibration is off by 2%, clean and recalibrate.
- Monitor the lot number of the used reagents. Use the same lots while setting up the assay to exclude slight variations among lots as the source of experimental variation.
- Have backup-plans (e.g.: excess plates) and always know what you are doing in case of an unlikely, but possible, problem (e.g.: software error on robot). **If there is a delay or problem in the assay, always discard the plate and do a new experiment.**
- When doing control experiments for setting up the assay, use computer vision to correctly quantify the RNA spots and detect subtle variations among individual wells.

SUGGESTED CONTROLS FOR SETTING UP IMAGE-BASED TRANSCRIPTOMICS

- Add several control genes with different expression levels. We use dapB (bacterial gene with absent expression), erbb2 (low expression), hprt1 (medium expression) and actb (high expression).
- Perform tests, where each aspiration, pipetting, mixing step is performed in excess and ensure that no cells become detached. Short live cell imaging at low magnification with Hoechst dye immediately before the FISH assay allows you to ensure that you do not wash any cells away.
- Perform multiple identical experiments on **different days** to ensure high technical and biological reproducibility.
- Have a set of control experiments, where you stain all wells of a plate with the same probes. Ensure the number of spots per cell is not variable among wells (e.g.: difference towards certain borders of the plate or according to processing scheme of your liquid handling platform).
- To ensure that your microscopic acquisition is sensitive enough, use special probesets, where only a single epitope of a given transcript (recommended hprt1) is targeted. You should still see specific spots (see Battich et al. 2013).
- Dual labelling of the same RNA (hybridization efficiency) and comparison with RNAseq (Battich et al. 2013)

ADJUSTMENTS THAT MAKE YOUR ASSAY UNRELIABLE

- Other incubators will lead to positional effects (see above).
- Shorter incubation times and/or preheated washbuffer (at hybridization temperature) should be avoided. The assay will become less robust and therefore less reproducible between multiple replicate weeks. While we would still be seeing a strong difference between positive and negative controls, these adjustments can favour positional bias. The arising problems can impact certain probesets/genes more than others and are therefore not suitable for image-based transcriptomics with 1000s of different genes.
- Oligo-nucleotide single molecule FISH reagents (e.g.: Stellaris FISH) are not suitable for transcriptomics as performed here (see also Suppl. Fig 2 in Battich et al. 2013).

ADJUSTMENTS THAT CAN BE USEFUL

- Different cell lines can require different protease concentration for optimal reproducibility and sensitivity. Test assay with a protease dilution series when starting a new cell line.
- Add acetic acid during fixation to detect nuclear RNAs (Battich et al. 2013). Use SNORD3 as positive control. During the setup of your assay, use increasing acetic acid concentrations to show that any variability in nuclear RNA detection between single cells is not an artefact from variability in the accessibility of different nuclei (which we observed at intermediate acetic acid concentrations). Also monitor cytoplasmic RNA to ensure that cytoplasmic RNA is not lost (if you want to study it too).

LIQUID HANDLING

- Dispense to the right side of the well, close to the well border.
- Aspirate in middle of well.
- Pipet/Mix in middle of well.
- Use tip-touch to ensure even pipetting.
- Use slow aspiration / dispensing / pipetting speeds.
- When using new reagents in the peristaltic pump: (Clean with Water) / Prime / soak / purge / prime / purge /prime

EXAMPLE PROTOCOL

Experiment Title:

Date:

Notes

1	This protocol assumes processing of three 384-well plates in sequential order.
2	Every aspiration step was done in the EL406 BioTek washer-dispenser so that 15ul residual volume were left in the well.
3	Tips used for dispensing and mixing with Bravo platform were 70ul Tips (# 19133-212). Tips were freshly discharged with a Milty Zerostat3 not more than 1h before immediate use.
4	This protocol is based upon Affymetrix's protocol: QuantiGene ViewRNA HC Screening User Manual, RevD
5	Few remaining manual steps could also be fully automated.
6	PreHyb plates can be refilled during the assay. This refilling is not indicated. Prepare two plates of PreHyb to reduce effect of unexpected problems.
7	Prepare protocols for machines before starting the assay (and double check processed volumes manually).
8	For fixation, fix all wells of the plate (not only the actually used wells)
9	Succinimidyl ester staining is best, when succinimidyl ester is added to carbonate buffer within 30sec prior to use. Consider separate batches of succinimiyl ester solution for individual plates.

Abbreviations

RT	Room Temperature
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
SuccEst	Alexa Fluor® 647 carboxylic acid, succinimidyl ester (Invitrogen)

Step	Hour	Min	Plate	Name	Description	Check	Comments
1			N/A	Reagents	Warm Reagents: PreHyb,PS_Diluent, Amp_Diluent, LP_Diluent,PBS (for PreHyb plates only) for 30min 40C		
2			N/A	Reagents	Prepare 8% PFA ad leave at RT.		
3			N/A	Reagents	Prepare Wash Buffer ad leave at RT.		
4			N/A	Reagents	Prepare Probe Sets leave at RT.		
5			N/A	Reagents	Make sure PreHyb is at 40C. Prepare 2 plates and store at 40C.		
6			N/A	Reagents	Prepare Pre Amp leave at RT.		
7			N/A	Reagents	Prepare Amp leave at RT.		
8			N/A	Reagents	Prepare Label Probe leave at RT.		
9			N/A	Equipment	Start **Biotek EL406** and test functionality, 1ul Cassette		
10			N/A	Equipment	Start **Bravo** (and **initialize Liconic)**		
11			N/A	Break	Have Breakfast		
12			N/A	Reagents	Prepare Mitotracker in Serum Free Medium. Use within 1min.		OPTIONAL
13			All	Mitotracker	Aspirate medium, dispense **Mitotracker** (15ul), Incubate@37C for 45 min.		OPTIONAL
14			All	Cell Fixation	Wash 2x with PBS (80ul), fix plate with **8% PFA** (15ul) for 30 min at RT.		
15			All	Cell Fixation	Aspirate PFA and wash 3x with PBS.		
16			N/A	Equipment	Remove **First and Last tubes** of Biotek EL406 1ul cassette.		
17			1	Cell Perm.	Aspirate PBS, dispense **Detergent Solution** (15ul) and incubate for 3 min at RT.		
18			1	Cell Perm.	Wash 2x with PBS.		
19			1	Protease	Aspirate PBS, dispense **Working Protease** (15ul), incubate 10 min at RT with lid closed.		
20			1	Protease	Wash 5x with PBS.		
21			1	Protease	Aspirate PBS, dispense **Protease Stop Buffer** (15ul).		
22			1	Protease	Mix by pipetting up and down twice		
23			1	Protease	Aspirate **Protease Stop Buffer**, dispense Protease Stop Buffer (15ul).		
24	0	0	1	Probe Set	Dis pence **Working Probe Set** with Bravo (15ul). Place Plate in incubator		
25	0	10	2	Cell Perm.	Aspirate PBS, dispense **Detergent Solution** (15ul) and incubate for 3 min at RT.		
26			2	Cell Perm.	Wash 2x with PBS.		

27			2	Protease	Aspirate PBS, dispense **Working Protease** (15ul), incubate 10 min at RT with lid closed.		
28			2	Protease	Wash 5x with PBS.		
29			2	Protease	Aspirate PBS, dispense **Protease Stop Buffer** (15ul).		
30			2	Protease	Mix by pipetting up and down twice		
31			2	Protease	Aspirate **Protease Stop Buffer**, dispense Protease Stop Buffer (15ul).		
32			2	Probe Set	Dispense **Working Probe Set** with Bravo (15ul). Place Plate in incubator		
33	0	50	3	Cell Perm.	Aspirate PBS, dispense **Detergent Solution** (15ul) and incubate for 3 min at RT.		
34			3	Cell Perm.	Wash 2x with PBS.		
35			3	Protease	Aspirate PBS, dispense **Working Protease** (15ul), incubate 10 min at RT with lid closed.		
36			3	Protease	Wash 5x with PBS.		
37			3	Protease	Aspirate PBS, dispense **Protease Stop Buffer** (15ul).		
38			3	Protease	Mix by pipetting up and down twice		
39			3	Protease	Aspirate **Protease Stop Buffer**, dispense Protease Stop Buffer (15ul).		
40			3	Probe Set	Dispense **Working Probe Set** with Bravo (15ul). Place Plate in incubator		
41	1	20	N/A	Equipment	Change Cassette of BioTek to 5ul Cassette		
42	1	25		Break	Lunch		
43	2	50	1	Probe Set	Aspirate **Working Probe Set**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
44			1	Pre Amp	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate PreHyb		
45			1	Pre Amp	Dispense **Working Pre Amp** (15ul) with Bravo by quadrant. Place plate in the incubator.		
46	3	40	2	Probe Set	Aspirate **Working Probe Set**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
47			2	Pre Amp	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate PreHyb		
48			2	Pre Amp	Dispense **Working Pre Amp** (15ul) with Bravo by quadrant. Place plate in the incubator.		

49	4	0	1	Pre Amp	Aspirate **Working Pre Amp**, do 3x Wash Buffer at 40C followed by 30 sec incubation.		
50			1	Amp	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate PreHyb		
51			1	Amp	Dispense **Working Amp** (15ul) with Bravo by quadrant.		
52	4	30	3	Probe Set	Aspirate **Working Probe Set**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
53			3	Pre Amp	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate PreHyb		
54			3	Pre Amp	Dispense **Working Pre Amp** (15ul) with Bravo by quadrant. Place plate in the incubator.		
55	4	50	2	Pre Amp	Aspirate **Working Pre Amp**, do 3x Wash Buffer at 40C followed by 30 sec incubation.		
56			2	Amp	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate PreHyb		
57			2	Amp	Dispense **Working Amp** (15ul) with Bravo by quadrant.		
58	5	10	1	Amp	Aspirate **Working Amp**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
59			1	LP	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate PreHyb		
60			1	LP	Dispense **Working Label Probe** (15ul) with Bravo by quadrant.		
61	5	40	3	Pre Amp	Aspirate **Working Pre Amp**, do 3x Wash Buffer at 40C followed by 30 sec incubation.		
62			3	Amp	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate PreHyb		
63			3	Amp	Dispense **Working Amp** (15ul) with Bravo by quadrant.		
64	6	0	2	Amp	Aspirate **Working Amp**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
65			2	LP	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate		

					PreHyb		
66			2	LP	Dispense **Working Label Probe** (15ul) with Bravo by quadrant.		
67	6	20	1	LP	Aspirate **Working Label Probe**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
68			1	LP	Wash 3x with PBS.		
69	6	50	3	Amp	Aspirate **Working Amp**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
70			3	LP	Aspirate Wash Buffer, dispense **PreHyb Buffer** (15ul) with Bravo by quadrant, pipette up and down twice, aspirate PreHyb		
71			3	LP	Dispense **Working Label Probe** (15ul) with Bravo by quadrant.		
72	7	10	2	LP	Aspirate **Working Label Probe**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
73			2	LP	Wash 3x with PBS.		
74			N/A	Reagents	Prepare DAPI.		
75	7	50	3	LP	Aspirate **Working Label Probe**, do 3x Wash Buffer at RT followed by 30 sec incubation.		
76			3	LP	Wash 3x with PBS.		
77	8	0	All	DAPI	Aspirate PBS, dispense **Working DAPI** reagent (70ul), incubate at RT for 10 min		
78			All	DAPI	Wash 3x with PBS.		
79	8	20	N/A	Reagents	Prepare SuccEst.		
80	8	30	All	SuccEst	Aspirate PBS, dispense **Working SuccEst** reagent (70ul), incubate at RT for 5 min		
81			All	SuccEst	Wash 3x with PBS.		
82	8	40	All	Cover	Cover all plates with sticky metal foil		
83	8	50	1	Microscope	Image plates (do not make new settings if tired)		

Have fun!

Highly recommended: Once have a look at the RNA particles in a normal epifluorescence microscope by looking at them through the eyepiece. Branched DNA single-molecule FISH is so bright that you can easily see single RNA molecules with your own eyes. This is absolutely beautiful.